IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Moser, et al.	Group Art Unit: 1644
Appl. No.	:	09/802,397	
Filed	:	March 9, 2001	
For	:	**DENDRITIC-LIKE CELL/TUMOR CELL HYBRIDS AND HYBRIDOMAS FOR INDUCING AN ANTI-TUMOR RESPONSE**	
Examiner	:	Ewoldt, Gerald R.	

SECOND DECLARATION OF DR. MURIEL MOSER UNDER 37 C.F.R. § 1.132
(MOSER II)

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Muriel Moser, declare as follows

1. I am an inventor in the above-identified application.

2. The following experiments evidence that proliferating DCs provide better fusion partners for DC/tumor cell fusions and that the DC/tumor cell fusions formed are capable of providing immunostimulation. First, the phenotype of bone marrow progenitors at different times of culture was characterized (in presence of GM-CSF) and proliferation was monitored. The data is shown in Figures 1-3 attached. Figures 1-2 show evolution of several markers over the 9 day culture period: MHC-II, CD11c, F4/80, GR1 (Ly6G), CD90.2, CD4, CD8, and B220/CD45R. These results show that dendritic cells derived from bone marrow (BMDCs) are CD11c, MHC-II and F4/80 positive and 50% are GR1 positive. These BMDCs do not express B-cell or T-cell

markers (except CD90.2 at a negligible level). DC specific markers occur early in culture at day 2. 90% of cells are CD11c positive at day 4 and MHC-II positive at day 6.

3. In order to follow cell divisions, progenitor cells were labeled at D0 with CFSE, a cytoplasmic dye which divides equally into daughter cells and therefore decreases proportionally to cell division. The FACS analysis (Figure 3) shows the decrease of CFSE labeling over the course of culture. Cells were also stained for CD11c, MHC-II, F4/80, GR1, B220, CD4 and CD8 molecules. Cell divisions occurred mainly during the first 3 days of culture as shown by the left shift of CFSE staining. The rate of division seems to decrease significantly at day 4, which correlated with the expression of CD11c and F4/80 on almost all cells. At day 3, the high proportion of FL2 negative cells suggested that progenitor cells are dividing and remain poorly differentiated. Thus, cells at day 3 are proliferating. Conversely, at day 4, most cells are CD11c, F4/80, and to a lesser extent, MHC-II and GR1 positive.

4. Fusion experiments were performed using the BMDCs characterized above in paragraphs 2-3. Fusion of early BMDCs (days 3 and 4 of culture) was compared to fully differentiated BMDCs (day 9 of culture). HAT sensitive P815 tumor cells and BMDCs harvested at day 3, 4 or 9 of culture (end of culture) were mixed at 1:1 ratio (10^7 BMDCs and 10^7 P815), washed in serum free DMEM medium at 37°C and spun down. The cell pellet was broken by gentle agitation with a 2 ml serological pipet in presence of 500 µl of PEG 1500 (Boeringher Mannheim). 500 µl of DMEM medium (37°C) were added 1.5 min later. Increasing volumes of DMEM (1, 2, 4 ml) were added every 1.5 min. Cells were then spun down, diluted in PBS BSA (1%)-EDTA (10 mM) and sorted by magnetic microbeads linked to anti-CD11c antibodies, diluted in complete HAT medium and plated. After 24 hours of culture, hybrids were cloned by limiting dilutions in 96 well plates at 0,3 and 1 cell per well in complete HAT medium.

5. ***Fusion yield***

The table shows that higher numbers of hybrid cells were obtained after fusion between P815 and BMDCs at day 3 of culture than with BMDCs at days 4 or 9 of culture, confirming that proliferating DCs are more efficient in DC/tumor cell fusions.

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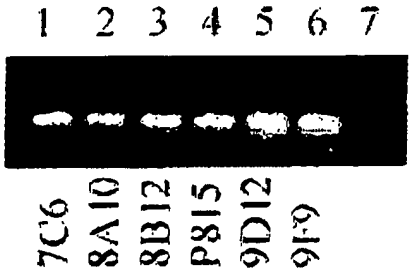
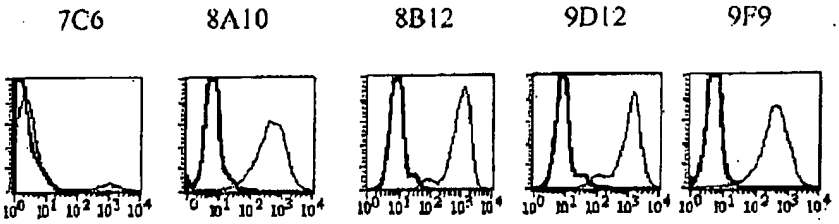
	Number of clones obtained with BMDCs at day 3 of culture in 3X96 well plates (1 cell/well)	Number of clones obtained with BMDCs at day 4 of culture in 3X96 well plates (1 cell/well)	Number of clones obtained with BMDCs at day 9 of culture in 3X96 well plates (1 cell/well)
Exp 1	54	12	-
Exp 2	28	5	1
Exp 3	135	5	-

6. ***Phenotype analysis of hybrid cells***

The fused cells were analyzed to determine that they were true hybrids between DCs (defined as CD11c positive) and P815 tumor cells. CD11c expression was analyzed by flow cytometry and expression of mRNA specific for P815-associated antigen P1A was assessed by RT-PCR. Among 27 clones (exp 3), 26 were CD11c+ and 19 were P1A+.

Example of phenotype analysis for 5 clones





mRNA expression of P1A is shown for the five clones (lanes 1, 2, 3, 5, 6), P815 tumor cells (lane 4) and 3B4 irrelevant cell, the negative control (lane 7). All clones express tumor antigen P1A. The FACS analysis shows that four clones express CD11c.

7. We conclude that fusion of P815 tumor cells and BMDCs cultured for only 3 days, while the DCs are proliferating, produces more hybrid cells than fusion with BMDCs cultured from 4 or 9 days. Thus, we conclude that it is preferable to use proliferating dendritic cells to make DC/tumor cell fusions because the yield of fused cells is much higher when proliferating DCs are used.

8. Allo-MLRs were performed to assess the immunostimulatory properties of hybrid cells. Hybrid cells (I-Ad) treated or not with LPS (50 µg/ml overnight) were cultured with purified naive T cells (I-Ab). Results from testing of 19 P1A+/CD11c+ hybrid clones:

2 exhibit poor immunostimulatory properties

4 exhibit weak immunostimulatory properties

13 exhibit strong immunostimulatory properties

We conclude that DC/tumor cell fusions may be produced efficiently using proliferating DCs isolated from bone marrow and that these fused cells have strong immunostimulatory properties.

9. We also conducted experiments using different sources for isolation of DCs. While paragraphs 1-8 above describe procedures using DCs from bone marrow, sources such as blood, lymph, lymph nodes and spleen may also be used. We have found that bone marrow, blood and lymph contain a low number of differentiated DCs, but a high number of DC progenitors. Spleen and lymph nodes contain a high number of differentiated DCs and a low number of DC progenitors. As demonstrated in the instant specification, DCs isolated from spleen produced a T-cell/tumor cell hybridoma, not a DC/tumor cell hybrid (see Examples 1-6 and paragraph 0184 of the published application). Further experiments in my laboratory have confirmed that spleen is a poor source for DCs to produce DC/tumor cell hybrids while bone marrow, blood and lymph are the preferred sources.

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10. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States codes and that such willful, false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: February 4, 2005 By: Muriel Moser

Muriel Moser

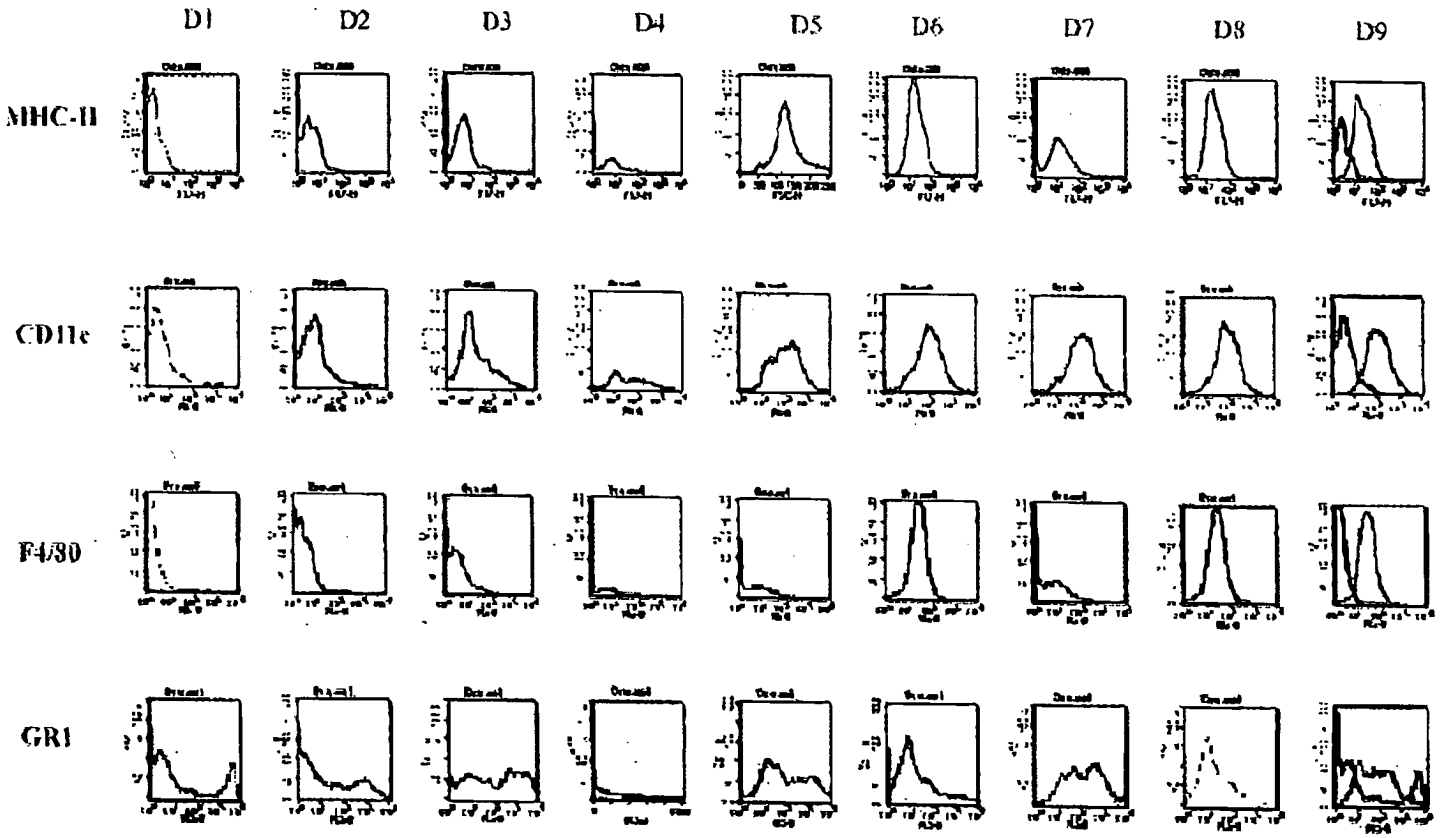

FIGURE 1

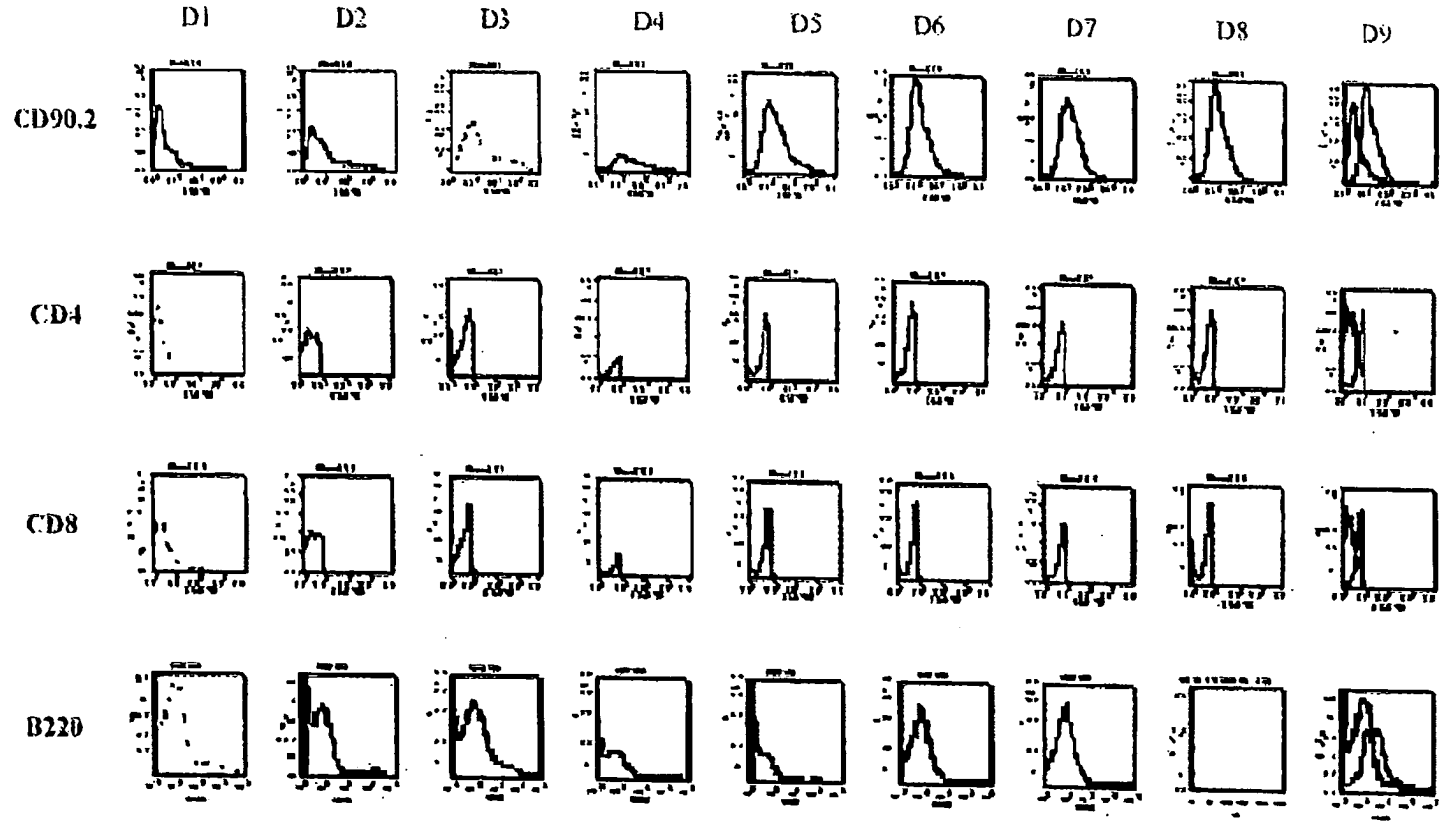

FIGURE 2

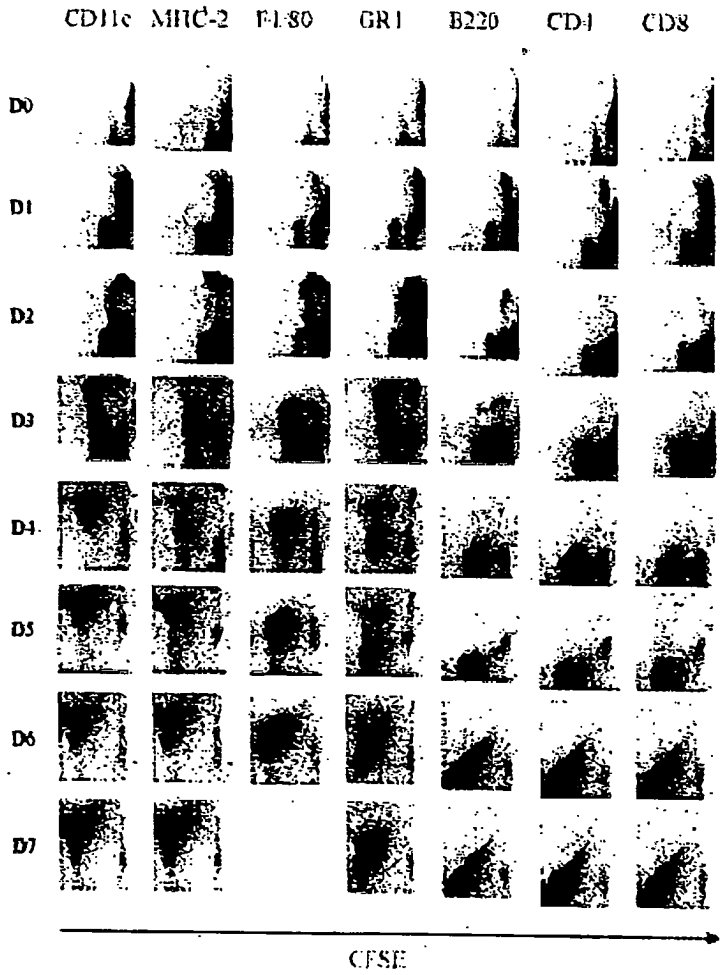

FIGURE 3